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# Activity of Polymerase Proteins of Vaccine and Wild-Type Measles Virus Strains in a Minigenome Replication Assay

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**The relative activities of five measles virus (MV) polymerase (L) proteins were compared in an intracellular, plasmid-based replication assay. When coexpressed with N and P proteins from an attenuated strain, L proteins from two attenuated viruses directed the production of up to eight times more reporter protein from an MV minigenome than the three wild-type L proteins. Northern blot analysis demonstrated that the differences in reporter protein production correlated with mRNA transcription levels. Increased activity of polymerases from attenuated viruses equally affected mRNA transcription and minigenome replication. The higher level of transcription may be a consequence of increased template availability or may be an independent effect of the elevated activity of the attenuated polymerases. Coexpression of wild-type L proteins with homologous N and P proteins did not affect the activity of the wild-type polymerases, indicating that the differential activity was a function of the L proteins alone. Use of a minigenome that incorporated two nucleotide changes found in the genomic leader of the three wild-type viruses did not raise the activity of the wild-type L proteins. These data demonstrate that increased polymerase activity differentiates attenuated from wild-type viruses and suggest that functions involved in RNA synthesis contribute to the attenuated phenotype of MV vaccine strains.**

*Measles virus* (MV) is a member of the *Morbillivirus* genus of the family *Paramyxoviridae*. Its single-stranded, negative-sense genome encodes three structural proteins that are involved in transcription and replication of the viral genome, the nucleoprotein (N), the phosphoprotein (P), and the polymerase or large (L) protein (for reviews, see references 15 and 21). The N protein (525 amino acids [aa]) encapsidates the viral genome and antigenome. This nucleocapsid serves as the template for the viral polymerase complex, which consists of the L and P proteins. It is assumed that the L protein (2,183 aa) contains all of the enzymatic activities required for transcription and replication. In addition to its role as a cofactor in the polymerase complex, the P protein (507 aa) also acts as a chaperone in nucleocapsid assembly (19). The P mRNA contains a second open reading frame, which is translated into the nonstructural C protein (3). Approximately 50% of the P mRNAs are modified through the addition of a single, nontemplated nucleotide at position 751. This process, termed RNA editing, is a function of the viral polymerase and results in translation of the nonstructural V protein (6). Both the C and the V proteins are dispensable for virus growth in Vero cells (32, 39), but they may play a role in pathogenesis (11, 30, 45, 47). RNA synthesis requires additional cellular factors, only one of which (tubulin) has been identified (25). All of the *cis*-acting elements necessary for transcription and replication (i.e., promoters and encapsidation signals) are located in the termini of the MV genome (40). Replication is initiated in both the genomic and antigenomic leader sequences. The promoter(s) in the genomic leader also direct the sequential transcription of the six MV genes.

Live, attenuated MV vaccines provide safe and effective prevention of disease; however, the genetic basis for their attenuation has yet to be determined. Attenuation was achieved by adaptation of wild-type MV isolates to cell culture by using a range of cell lines and culture conditions (36). Several vaccine strains are licensed in different countries, and most of these strains were derived from the Edmonston wild-type isolate described by Enders (10). On the basis of phylogenetic analysis, wild-type MV isolates can be divided into 20 genotypes (48). All MV vaccine strains and a number of wild-type isolates, including the prototype Edmonston wild-type strain, are grouped into genotype A.

A comparison of the predicted amino acid sequences of several MV vaccine strains with their Edmonston wild-type progenitor had suggested that amino acid changes in the viral proteins involved in RNA synthesis might play a role in attenuation (29). However, sequencing of the L genes of four MV vaccine strains and six wild-type isolates representing four genotypes had not identified amino acid substitutions that consistently distinguished vaccines from wild-type viruses (2). Nevertheless, since every vaccine strain has its unique passage history, it is possible that each may have acquired a unique set of attenuating mutations at different positions of the genome. Therefore, functional assays are required to analyze the role of sequence variations between vaccine strains and wild-type viruses.

To address the question of whether polymerase proteins contribute to an attenuated phenotype, we compared the relative replicative activities of three polymerases of wild-type viruses with two from attenuated strains. A plasmid-based MV minireplicon that expresses chloramphenicol acetyltransferase

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(CAT) (40) was used as the template in an intracellular replication assay. We found that minigenome replication and reporter gene transcription by polymerases cloned from attenuated viruses were increased up to eightfold over the levels produced by the L proteins of wild-type viruses. Coexpression of homologous N and P proteins did not affect the activity of wild-type L proteins. A minigenome with mutations in the genomic leader sequence that are found in the three wild-type viruses did not increase the activity of wild-type polymerases. The elevated activity of the polymerases of attenuated strains suggests that amino acid substitutions in the L proteins may play a role in attenuation.

## MATERIALS AND METHODS

**Cells and viruses.** Vero cells, CV-1 cells, and primary chicken embryo fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, and antibiotics. The three wild-type MVs MVi/Bilthoven.NET/91 (Ne), MVi/Chicago.USA/89/1 (Il-1), and MVi/Banjul .GAM/91/1086 (Gam) and the Kitasato submaster seed of the CAM-70 strain (kindly provided by I. Yoshida, Kanonji Institute, Osaka, Japan) were propagated in Vero cells. The MVAT7 recombinant vaccinia virus, which expresses the T7 RNA polymerase, was kindly provided by B. Moss, Bethesda, Md., and was propagated in primary chicken embryo fibroblasts.

**RNA preparation, RT-PCR, and sequencing.** Total cellular RNA was extracted from MV-infected Vero cells by the guanidinium-acid-phenol method (7). Reverse transcription (RT) reactions were performed with an oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies) in accordance with the manufacturer's recommendations. PCR was performed with the Elongase enzyme mix (Life Technologies) by using the manufacturer's recommendations for long RT-PCR. The CAM-70 *L* gene was amplified in two fragments as described previously (2). The generation of the Ne, IL-1, and Gam *L* clones has been described previously (2). The *P* genes of CAM-70, Ne, Il-1, and Gam were amplified with *P*-specific primers from the same RNA preparation used to clone the respective *L* genes by using the same RT-PCR procedure as described above. The *P*-specific primers were 5′-gatcgaattccgATGGCAGAAGAGCAGGCACGCCA**C**GTCA-3′ (primer 1) and 5′-gatcctcgagGCTGTAGCTACTTCAT-3′ (primer 2). The *Eco*RI and *Sal*I restriction sites that were used to insert the *P* genes into pTM1 are underlined. Uppercase nucleotides denote MV sequences. The nucleotide highlighted in bold in primer 1 changed the C start codon from ATG to ACG. The EdmBil *P* gene was PCR amplified from MV infectious clone p(+)MV (a generous gift of M. Billeter, Zürich, Switzerland) with the same primers used for the other *P* genes. PCR fragments were sequenced with the ABI PRISM Dye Terminator reaction kit (Perkin-Elmer) in accordance with manufacturer's instructions with sequencing primers based on the EdmBil sequence (GenBank accession number Z66517 [33]). Sequence data were analyzed with the ABI PRISM Autoassembler DNA sequence assembly software (Perkin-Elmer) and version 10.0 of the sequence analysis software package of the University of Wisconsin Genetics Computer Group (9). For analysis of conservative amino acid changes, amino acids were grouped into families as follows: Phe-Tyr, Met-Leu-Ile-Val, Ala-Gly, Thr-Ser, Gln-Asn, Glu-Asp, Lys-Arg, His, Pro, Trp, and Cys.

**Construction of plasmids.** A full-length clone of CAM-70 *L* was assembled in pTM1 (24) by using the *Bam*HI and *Sal*I sites, as well as the internal *Sac*I site at nucleotide 3417 in the *L* coding sequence. Where necessary, nucleotides that deviated from the consensus sequence were repaired by use of the Excite mutagenesis kit (Stratagene). Our goal was to clone all *L* genes into the same expression vector with identical cloning strategies and 5′ noncoding regions. This required modification of the Il-1 and EdmBil L clones. Since the Il-1 *L* gene had been amplified with a different set of primers (2), it lacked the 5′ noncoding region. To correct it, a 400-bp fragment of the 5′ end of the Il-1 *L* gene was amplified from the pTM1-Il-1 *L* plasmid with 5′-atgggatcc**GGTCCAAGTGGTTCCCCGTT**ATGGACTCGCTATCT-3′ (primer 3) and 5′-GTTAGTGTCCCTTAAG-3′ (primer 4). The *Bam*HI restriction site that was used to insert the *L* gene into pTM1 is underlined. The nucleotides highlighted in bold contain the 5′ noncoding region of the *L* gene. After digestion with *Bam*HI and *Bsu*36I (which cuts at nucleotide 382 in the Il-1 *L* coding region), the fragment was used to replace the corresponding fragment of the pTM1-Il-1 *L* clone. The EdmBil *L* gene was subcloned into pTM1 from pAeL, a gift of M. Billeter. The 5′ end of the pTM1-EdmBil *L* clone could be generated with the same 5′ fragment that was used for Il-1 *L* since the first 100 aa of EdmBil L and Il-1 L are identical. A three-way ligation was performed with pTM1 (digested with *Bam*HI), the 5′ fragment (digested with *Bam*HI and *Bbs*I, which cuts at nucleotide 299 in the *L* coding region), and the remainder of the EdmBil *L* gene cut out of pAeL with *Bbs*I and *Bam*HI. Resequencing of RT-PCR fragments of the Gam *L* sequence had revealed three nucleotide errors in the previously published sequence (2). The corrected sequence was submitted to the GenBank database. The pTM1-Gam *L* clone was repaired with the Excite Mutagenesis kit (Stratagene) to represent the consensus sequence.

All *P* genes were cloned into pTM1, with the *Eco*RI and *Xho*I sites. All *N* genes were subcloned from pBluescript KS into pTM1. The EdmBil *N* gene (a gift of K. Baczko, Würzburg, Germany) was subcloned into the *Bam*HI site. For the Ne *N* gene, *Sac*I and *Bam*HI were used, and for the Gam and Il-1 *N* genes, *Sac*I and *Xho*I sites were used. pMV107(−)CAT (40) was a gift of M. Billeter. It contains 107 nucleotides of the MV genomic 3′ end and 109 nucleotides of the genomic 5′ end flanking the open reading frame of the *cat* gene in a bluescript vector. The puc26A42G construct encodes an MV minigenome identical to that of pMV107(−)CAT, while in the puc26U42U construct, two nucleotides in the genomic leader have been mutated (X. Liu, unpublished data). The DNA concentrations of CsCl-purified plasmid preparations were determined with the fluorochrome Hoechst 33258 (14). Fluorescence was measured in a Spectrafluor fluorometer (Tecan). All *L* clones were resequenced after plasmid preparation to verify the correctness of the inserts.

**Protein expression.** CV-1 cells in 35-mm-diameter dishes were infected with MVAT7 at a multiplicity of infection of 5 and transfected simultaneously. Five micrograms of plasmid DNA in Opti-MEM medium (Life Technologies) was transfected with pfx6 (Invitrogen) in accordance with the manufacturer's recommendations. Cells were labeled from 6 to 10 h after transfection with [$^{35}$S]methionine in methionine-free medium (ICN). Cytoplasmic cell extracts were prepared in NET-BSA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, 1 mg of bovine serum albumin per ml, pH 7.4). Aliquots of cell extracts were incubated with N- or P-specific rabbit antisera, followed by precipitation with GammaBind G-Sepharose (Amersham Pharmacia Biotech). The complexes were washed with NET-BSA and separated by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis. After electrophoresis, bands were visualized by autoradiography.

**Replication assay.** CV-1 cells in 35-mm-diameter dishes were infected with MVAT7 at a multiplicity of infection of 5 and transfected simultaneously. A mixture of pTM1-N (1.55 μg), pTM1-P (0.45 μg), pTM1-L (0.40 μg), and pMV107(−)CAT (3.30 μg) in Opti-MEM medium (Life Technologies) was transfected with pfx6 (Invitrogen) in accordance with the manufacturer's recommendations. In negative controls, one of the four plasmids was replaced with pTM1. For every gene whose role was analyzed in an experiment (e.g., the *L* genes in Fig. 2), two plasmid preparations were used, and for every sample, transfection mixtures were set up in duplicate. Every experiment was carried out at least three times. At 28 h after transfection, 10 μg of actinomycin D (actinomycin D-mannitol; Sigma) per ml of medium was added to the medium.

**CAT ELISA.** Cytoplasmic extracts were prepared 42 to 48 h after transfection in 1 ml of lysis buffer (part of the CAT enzyme-linked immunosorbent assay [ELISA] kit [Roche]). The total protein concentration of the extracts was measured with the BCL kit (Pierce) by the microwell plate protocol, and optical densities were determined at 560 nm in a Rainbow ELISA reader (Tecan). The concentrations of CAT protein in the extracts were measured with the CAT ELISA kit (Roche), and optical densities were determined in the Rainbow ELISA reader at 405 nm. The amounts of extracts used per sample were adjusted for protein concentration to ensure comparable results.

**Northern blot analysis.** RNA was purified at 42 to 48 h after transfection. Poly(A)$^+$ RNA was isolated directly from cell lysates by use of Oligotex particles and buffers (Qiagen) in accordance with the manufacturer's recommendations for direct purification from cultured cells. The protocol was modified for a batch procedure and followed by ethanol precipitation. For analysis of minigenome replication, cytoplasmic extracts were treated with micrococcal nuclease (S7 nuclease; Roche) (12). In brief, cells were lysed in 200 μl of MN buffer (10 mM Tris-HCl [pH 7.5], 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM $CaCl_2$, 9,000 Kallkrein inhibitor units of aprotinin/ml [Roche], 200 U of micrococcal nuclease/ml). Incubation of the lysate at 30°C for 30 min was followed by RNA extraction with Trizol LS (Life Technologies) in accordance with the manufacturer's recommendations.

RNA extracted from one-fifth of a 35-mm-diameter dish was separated by electrophoresis on a 1.5% agarose gel containing 3.7% formaldehyde, transferred to a nylon membrane (Roche) by vacuum blotting (Bio-Rad), and fixed by UV cross-linking (Stratagene). Templates for in vitro transcription of digoxigenin (DIG)-labeled riboprobes corresponding to 380 bases of the *cat* gene were

TABLE 1. Comparison of deduced amino acid sequences of the L proteins of the Edmonston wild-type isolate, EdmBil, and CAM-70

| Amino acid | Ed wt[a] | EdmBil L | CAM-70 L | Comment |
|---|---|---|---|---|
| 205 | I | I | V | Before domain 1[b] |
| 214 | S | S | **N** | Domain 1 |
| 235 | I | I | V | Domain 1, highly conserved area |
| 429 | E | D | E | Between domains 1 and 2 |
| 723 | Y | Y | **S** | Domain 3 |
| 1091 | R | R | K | Domain 4 |
| 1629 | R | **Q** | R | Between domains 5 and 6 |
| 1717 | D | **A** | **A** | Between domains 5 and 6 |
| 1805 | N | **S** | N | Domain 6 |
| 1887 | N | **D** | **D** | After domain 6 |

[a] Single-letter amino acid code; bold letters denote nonconservative substitutions relative to the Edmonston wild-type (Ed wt) sequence.

[b] Locations of domains: 1, aa 214 to 408; 2, aa 495 to 599; 3, aa 653 to 876; 4, aa 926 to 1092; 5, aa 1129 to 1376; 6, aa 1754 to 1831 (31).

generated by PCR with primers incorporating the T7 promoter to generate positive- or negative-sense probes. In vitro transcription of the probes, hybridization, and detection of the bands were carried out with the DIG system (Roche). Signals were visualized by autoradiography or quantitated with a Fluorochrom 8000 light imaging system and software (Alpha Innotech Corporation). To prevent saturation of the stronger signals, the amounts of RNA loaded were adjusted for the expected signal intensity.

**Statistical analysis.** The CAT protein concentrations generated by the L proteins of CAM-70 and EdmBil were compared with those generated by the L proteins of the wild-type viruses. The results of the CAT ELISA were not normally distributed, so nonparametric analysis was used (22). Data were rank transformed for the comparison of every wild-type L activity with every attenuated L activity by a two-way analysis of variance with the experiments treated as blocks to take into account any difference between experiments (22, 41). The significance level for statistical analysis was 0.05.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *L* gene of Cam-70 and the *P* genes of Cam-70, Ne, Gam, and Il-1 have been deposited in the GenBank database under accession numbers AF462047 to AF462051.

## RESULTS

**Cloning and expression of MV *L* genes.** The wild-type Ne *L* (MVi/Bilthoven.NET/91), Il-1 *L* (MVi/Chicago.USA/89/1), and Gam *L* (MVi/Banjul.GAM/91/1086) genes had been cloned from viruses that represent genotypes C2, D3, and B2, respectively (2, 48). The EdmBil virus is an attenuated laboratory strain that was adapted to HeLa cell spinner culture (46) and used as the source material for the infectious clone. The EdmBil L protein was the only MV polymerase that had been previously shown to be active in functional assays (33).

The CAM-70 vaccine strain was derived from the Tanabe wild-type strain by passage in the chorioallantoic membrane of chicken embryos (27). The CAM-70 *L* gene sequence was determined by sequencing of RT-PCR products. Comparison of the deduced amino acid sequences of the L proteins of CAM-70 and the genotype A reference strain, the Edmonston wild-type isolate, showed seven amino acid differences (Table 1). The sequence variability within the *L* genes of genotype A viruses is low compared with that of the *L* genes of the non-genotype A wild-type viruses, which differed from the Edmonston wild-type isolate at 15 to 24 amino acid positions (2). While the variable positions in most *L* genes of genotype A viruses were located in the carboxyl-terminal half of the protein (2), five of the seven changes in CAM-70 L were found in the amino-terminal half. A full-length clone of the CAM-70 *L* gene representing the consensus sequence was constructed. The CAM-70 *L* plasmid directed the expression of a protein of approximately 240 kDa (data not shown). Expression of the other four L proteins had been demonstrated previously (2).

**Comparison of L protein activities in a minigenome replication assay.** Plasmids carrying one of the five *L* genes were cotransfected with EdmBil *N* and EdmBil *P* plasmids and pMV107(−)CAT. At 42 to 48 h after transfection, cytoplasmic extracts were prepared and the concentration of CAT protein was measured by ELISA. To ensure that differences in CAT protein production were not the result of variations in the amounts of transfected *L* plasmids or the quality of the plasmid preparation, two independent plasmid preparations were tested for every *L* clone. The same approach was used for all subsequent experiments. A representative experiment is shown in Fig. 1A. The two attenuated L proteins (EdmBil L and CAM-70 L) reproducibly produced four (EdmBil L) to eight (CAM-70 L) times more CAT protein than did the most active wild-type L protein. The difference in activity between the EdmBil and wild-type L proteins is statistically significant, with a $P$ value of $<0.0001$.

**Analysis of transcription and replication by Northern blot assay.** Since production of the reporter protein is dependent upon the transcription of its mRNA, we compared the amounts of mRNAs by Northern blot analysis. Poly(A)$^+$-selected RNA was hybridized with negative-sense DIG-labeled riboprobes. In these experiments, the amount of transcript produced by the CAM-70 L protein was set at 100% (Fig. 2B). The CAM-70 and EdmBil L proteins transcribed 9 and 16 times more mRNA than did the most active wild-type polymerase. This result demonstrates that the differences in the amount of CAT protein reflect differences in transcriptional activity among the L proteins. To analyze minigenome replication, cell lysates were treated with micrococcal nuclease, which destroys unencapsidated RNA, followed by RNA purification and Northern blot analysis with a positive-sense DIG-labeled riboprobe to detect genomic sense RNA. The CAM-70 and EdmBil L proteins increased replication by 8- and 11-fold over the most active wild-type polymerase (Fig. 2A), indicating that replication followed the same pattern of activity as transcription.

**Cloning and expression of wild-type N and P proteins.** We wished to examine whether coexpression of homologous N and P proteins instead of the EdmBil N and P proteins would increase wild-type polymerase activity. The nucleotide sequences of the *P* genes of the Ne, CAM-70, Il-1, and Gam viruses were determined from RT-PCR products, and the genes were cloned into expression vector pTM1. A comparison of the deduced amino acid sequences with that of the Edmonston wild-type P protein is shown in Table 2. The two most divergent P proteins (Il-1 P and Gam P) differed by 30 aa (5.9%), and among all six P proteins, 10% of the amino acid positions were variable. A comparison of the CAM-70 P protein with Edmonston wild-type isolate P and EdmBil P demonstrated a considerable number of substitutions, even within genotype A. Most of the variable positions were located in the amino-terminal 231 aa of the protein (15% of the positions were variable), which constitute the shared part of P and V.

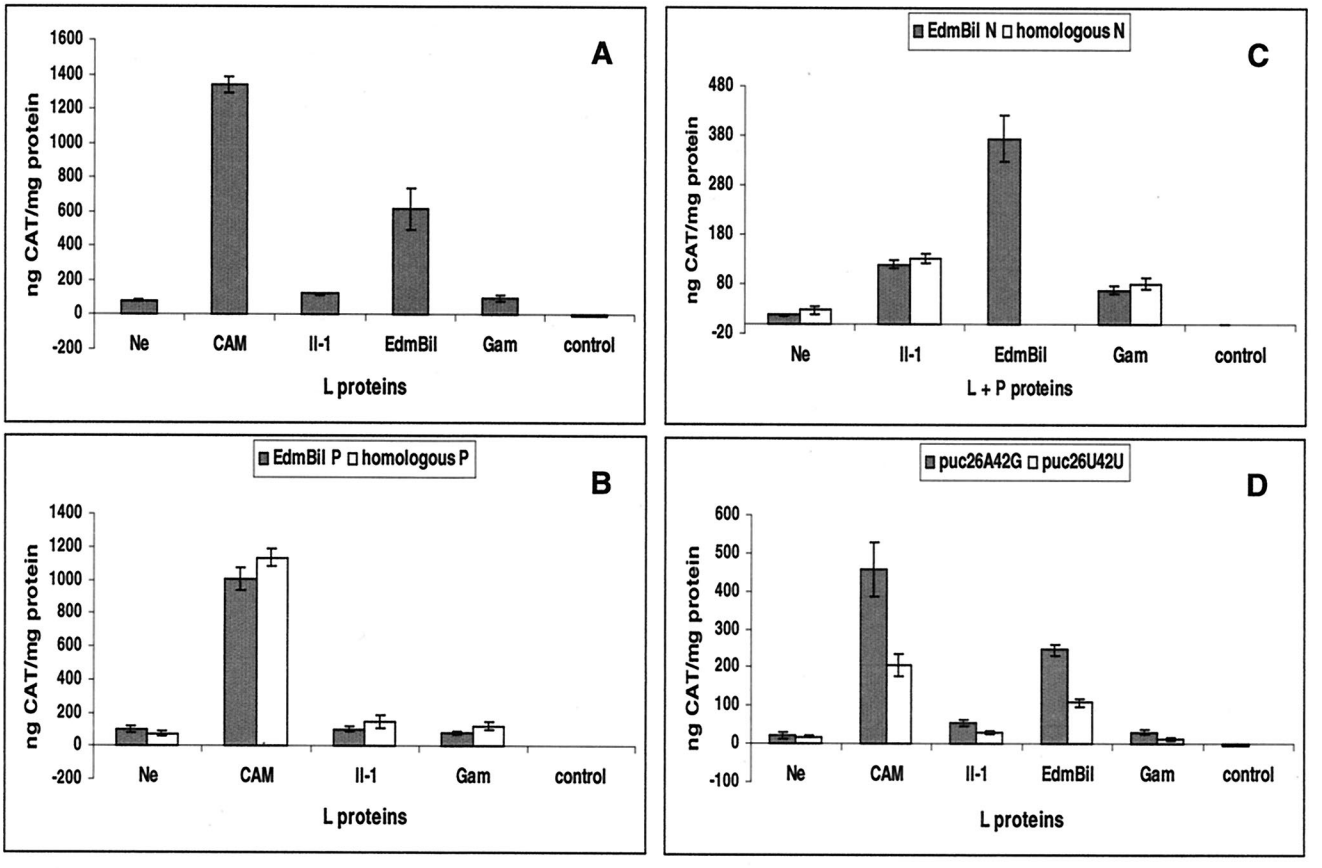


FIG. 1. Expression of CAT reporter protein by MV L proteins in a plasmid-based minireplicon assay. MVAT7-infected CV-1 cells were transfected with pTM1-EdmBil *N*, pTM1-EdmBil *P*, pMV107(−)CAT, and the indicated *L* plasmids (A); pTM1-EdmBil *N*, pMV107(−)CAT, the indicated *L* plasmids, and either pTM1-EdmBil *P* or the homologous *P* plasmid (B); pMV107(−)CAT, the indicated *L* plasmids with the homologous *P* plasmids, and either pTM1-EdmBil *N* or the homologous *N* plasmid (C); pTM1-EdmBil *N*, pTM1-EdmBil *P*, the indicated *L* plasmids, and either puc26A42G or puc26U42U (D). For the negative controls, one of the four plasmids was replaced with pTM1 as follows: the *L* plasmid in panel A, the *P* plasmid in panel B, the *N* plasmid in panel C, and the minigenome plasmid in panel D. Omission of any one of the four plasmids reduced the background to the same low level. CAT concentrations in cytoplasmic extracts were measured 42 to 48 h after transfection. Each panel shows the results of one experiment that is representative of three. In all of the experiments, the differences between the activities of the L proteins of attenuated strains and those of wild-type viruses were statistically significant, with a *P* value of $<0.0001$.

Amino acids 232 to 507 form the unique part of P and were somewhat more conserved (5% of the positions were variable), and almost half of these substitutions were conservative changes. The unique carboxyl terminus of the V protein (68 aa) and the C protein (186 aa) demonstrated the same level of substitution as the whole P protein (Table 3). Most of the changes in the C protein were located in the first 50 aa. It should be noted that the cloned *P* genes express neither C nor V proteins (see Materials and Methods).

The *N* gene sequences of the Ne, Il-1, and Gam viruses had been published previously (35, 37). These genes were subcloned into expression vector pTM1. To demonstrate that the plasmids directed the production of appropriately sized proteins, all N and P proteins were transiently expressed in MVAT7-infected CV-1 cells and immunoprecipitated with protein-specific antisera (Fig. 3). N and P proteins of different viruses demonstrated slight variations in their migration patterns in an SDS-polyacrylamide gel, a phenomenon described previously for other MV proteins (18, 38, 44). However, all of the proteins migrated within the expected size range, which suggests that they were synthesized correctly.

**Activity of wild-type L proteins in combination with homologous P and N proteins.** *L* plasmids were cotransfected with plasmids encoding the minigenome, EdmBil *N*, and either EdmBil *P* or the homologous *P* gene. One of three representative experiments is shown in Fig. 1B. The use of homologous P proteins did not increase the activity of wild-type L proteins. To examine the influence of homologous N proteins on wild-type L protein activity, homologous L and P proteins were coexpressed with either the EdmBil N or the matching N proteins. No significant change in wild-type L protein activity was observed when replication complexes utilized templates encapsidated in the homologous N protein (Fig. 1C). These results indicate that polymerase activity was not influenced by the source of the N and P proteins.

**A template with non-genotype A mutations in the leader sequence.** A recently published comparison of five vaccine strains and their wild-type progenitor had identified two nu-

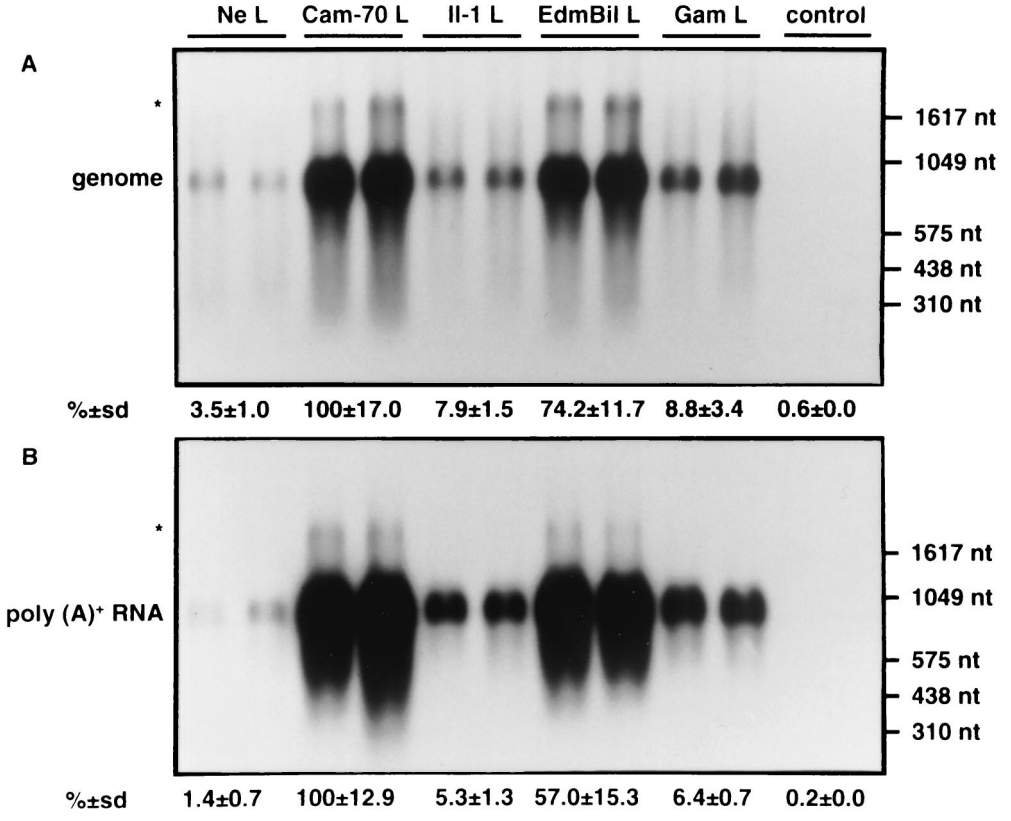


FIG. 2. Levels of transcription and replication by MV L proteins in the plasmid-based minireplicon assay. MVAT7-infected CV-1 cells were transfected with pTM1-EdmBil *N*, pTM1-EdmBil *P*, pMV107(−)CAT and the indicated *L* plasmids. Micrococcal nuclease-resistant genomic RNA was hybridized with a positive-sense probe (A). Poly(A)$^+$-selected mRNA was hybridized with a negative-sense probe (B). For the negative controls, the *L* plasmid was replaced with pTM1. Each panel shows one of three representative experiments. Molecular mass markers are shown on the right; the positions of genomic RNA and mRNA are indicated on the left. For both panels, RNA was separated by electrophoresis in formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with *cat* gene-specific, DIG-labeled riboprobes. For visualization by autoradiography, duplicate samples are shown and RNA extracted from one-fifth of a 35-mm-diameter dish was loaded onto the gel. For quantitation of band intensity, the amounts of RNA loaded were adjusted for expected signal strength to prevent saturation of the stronger signals. The signal produced by the CAM-70 L protein was defined as 100%, and the quantitative data are derived from quadruplicate samples. The asterisk denotes a background band that appeared primarily in lanes with strong signals, possibly as a result of incomplete denaturation of the sample. sd, standard deviation; nt, nucleotides.

cleotide positions in the genomic leader that distinguished the vaccine strains from the wild-type virus: positions 26 and 42 were A and G, respectively, in the vaccines but U in the wild-type progenitor (28). The 26U42U genotype was also found in the leader sequences of the Ne, Il-1, and Gam viruses, while the CAM-70 and EdmBil strains share the 26A42G leader (Liu, unpublished). In the course of another study, we had constructed two minigenomes, puc26A42G and puc26U42U. The first is a minigenome identical to that of pMV107(−)CAT, which was based on the EdmBil virus. Construct puc26U42U contains the two changes found in the leader sequences of the Ne, Il-1, and Gam viruses. The five *L* plasmids were cotransfected with plasmids encoding the EdmBil N and P proteins and either the puc26A42G or the puc26U42U minigenome. The puc26U42U template did not increase the activity of the wild-type polymerases (Fig. 1D). Instead, CAT production by all of the L proteins decreased significantly, with the exception of that of Ne L, which directed the production of comparable amounts of CAT protein from both templates. Similar results were obtained when the wild-type L proteins were coexpressed with their homologous N and P proteins (data not shown). These results demonstrate that changing nucleotides 26 and 42 of the genomic leader to the nucleotides found in wild-type viruses does not increase CAT production by wild-type polymerases.

## DISCUSSION

We compared the relative activities of five MV polymerases in an intracellular, plasmid-based replication assay as a first step toward characterizing the inherent properties of these multifunctional proteins. The polymerases from attenuated strains produced four- to eightfold more reporter protein than did the polymerases from wild-type MVs, and this increase in CAT production correlated with increased levels of transcription and replication. These results suggest that elevated levels of RNA synthesis differentiate the attenuated viruses from wild-type viruses and may be a factor contributing to the attenuated phenotype. Matching the wild-type L proteins with homologous N and P proteins in the replication assay did not

TABLE 2. Comparison of deduced amino acid sequences of the P proteins of attenuated and wild-type MVs

| Amino acid | Ed wt P[a] | EdmBil P | CAM-70 P | Ne P | II-1 P | Gam P |
|---|---|---|---|---|---|---|
| 29 | I[b] | | | | V | |
| 30 | E | | | **Q** | | |
| 44 | G | | | | | **R** |
| 46 | E | | | | D | |
| 49 | T | | | **A** | **A** | |
| 51 | R | | | K | K | **T** |
| 54 | K | | | | **E** | |
| 56 | G | | | **S** | | |
| 65 | L | | | | **F** | |
| 79 | R | | | | | **C** |
| 83 | P | | | **S** | **S** | **S** |
| 95 | I | | | | | **T** |
| 97 | P | | | **S** | **S** | |
| 98 | R | | **G** | **G** | | **G** |
| 99 | N | | **D** | | | |
| 105 | T | | | | | **A** |
| 109 | C | | | | | **R** |
| 110 | Y | **H** | **C** | | | |
| 111 | Y | | | | **H** | |
| 121 | K | | **E** | | | |
| 130 | M | | V | | | |
| 138 | D | | **G** | | | |
| 146 | N | | | | **D** | |
| 165 | I | | V | | | |
| 173 | I | | | | **T** | |
| 195 | R | | | | K | |
| 206 | L | | | | | **P** |
| 207 | G | | | **R** | | |
| 215 | P | | | **L** | | |
| 217 | D | | | | **N** | |
| 218 | P | | | **L** | | |
| 219 | G | | | | **S** | |
| 220 | R | | | | **M** | |
| 225 | E | **G** | **G** | | | |
| 228 | I | | | | V | |
| 246 | L | **S** | | | | |
| 259 | P | | | | | **S** |
| 266 | G | | A | | **D** | |
| 280 | A | | | **V** | | |
| 285 | W | | **R** | | | |
| 306 | Y | **H** | | | | |
| 355 | R | | | | | K |
| 377 | L | | | **F** | | **F** |
| 390 | I | | | | L | |
| 414 | V | | | | | I |
| 419 | L | | I | | | |
| 422 | M | | | I | | |
| 447 | M | | | | V | |
| 482 | M | | | | L | |
| 492 | N | | | | | **D** |
| No. of changes[b] | | 4 | 11 | 13 | 20 | 14 |

[a] Single-letter amino acid code: bold letters denote nonconservative substitutions relative to the Edmonston wild-type (Ed wt) sequence.
[b] Relative to Edmonston wild-type isolate.

TABLE 3. Comparison of deduced amino acid sequences of the C proteins and the unique carboxyl terminus of the V proteins of attenuated and wild-type MVs

| Amino acid | Ed wt[a] | EdmBil | CAM-70 | Ne | II-1 | Gam |
|---|---|---|---|---|---|---|
| C proteins | | | | | | |
| 10 | G | | | | | **E** |
| 13 | R | | K | K | | |
| 15 | S | | | | **N** | |
| 23 | R | | | | | K |
| 25 | L | | | | **P** | |
| 35 | T | | | | **I** | **I** |
| 39 | S | | | | T | |
| 41 | P | | | **L** | | |
| 44 | G | | | **R** | **R** | **R** |
| 45 | K | | | | | R |
| 73 | A | **V** | **V** | **V** | **V** | **V** |
| 78 | R | | | | K | |
| 88 | S | | | | | **P** |
| 103 | I | | V | | | |
| 131 | I | | V | | | |
| 147 | I | | T | | | |
| 166 | S | | | | **P** | |
| 186 | S | | | | | **C** |
| No. of changes[b] | | 1 | 5 | 4 | 8 | 8 |
| V proteins | | | | | | |
| 237 | S | | | | **G** | |
| 259 | T | | | | | **I** |
| 266 | R | | **S** | | | |
| 272 | C | **R** | | | | |
| 285 | V | | **A** | | | |
| 291 | Y | **H** | | | | |
| No. of changes[b] | | 2 | 2 | 0 | 1 | 1 |

[a] Single-letter amino acid code; bold letters denote nonconservative substitutions relative to the Edmonston wild-type (Ed wt) sequence.
[b] Relative to the Edmonston wild-type isolate.

increase their levels of activity to those of the polymerases from attenuated viruses, which indicated that the elevated levels of RNA synthesis were a function of the L proteins alone.

Before this publication, only one functional MV L clone had been described, that of laboratory-adapted Edmonston virus EdmBil (33). The CAM-70 vaccine strain was chosen for this study because it is an attenuated strain that was not derived from the Edmonston lineage. Sequence analysis demonstrated that the CAM-70 L protein is one of the most divergent L proteins within genotype A. Five of the seven amino acid differences between the L proteins of the Edmonston wild-type isolate and CAM-70 are unique to CAM-70 and are not present in any other L protein of genotype A viruses (aa 205, 214, 235, 723, and 1091). A comparison of the deduced amino

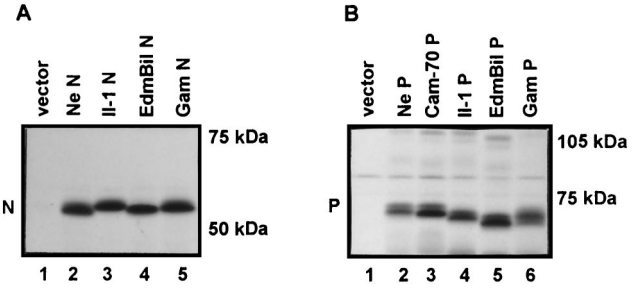


FIG. 3. Expression of MV N and P proteins. MVAT7-infected CV-1 cells were transfected with the indicated plasmids. $^{35}$S-labeled proteins were immunoprecipitated with N- or P-specific rabbit antisera and separated by SDS–12% polyacrylamide gel electrophoresis. Molecular mass markers are shown on the right; the positions of the N and P proteins are indicated on the left. Vector: cells transfected with pTM1 as a control. (A) Expression of N proteins; B, expression of P proteins. (B) A protein of approximately 90 kDa was precipitated nonspecifically in every lane.

acid sequences of the L proteins of five nonsegmented, negative-strand RNA viruses had defined six conserved domains, which may correlate with the various enzymatic activities (31). Within these domains lie shorter, more highly conserved amino acid sequences, some of which show homologies to known motifs, such as phosphodiester bond formation and nucleotide binding (4, 31). The hinge region between domains II and III is completely conserved in the L proteins of all MVs in genotype A, including CAM-70. However, this region is the least conserved region among L proteins of non-genotype A MVs (2), as well as other nonsegmented, negative-sense RNA viruses (31). Whether the conservation of the hinge region in the L proteins of MVs in genotype A has functional consequences is not known. An Ile-to-Val change at aa 235 in the L protein of CAM-70 is located in a highly conserved region of amino acids within domain I. However, the functional significance of this change is doubtful because the substitution in CAM-70 is conservative and Sendai virus, another paramyxovirus, also has Val at this position.

The MV minigenome in the vaccinia virus T7-driven replication assay is both transcribed and replicated (20). Northern blot assays of poly(A)$^+$-selected RNA verified that the amount of CAT protein correlated with the amount of CAT mRNA produced, while Northern blot analysis of micrococcal nuclease-treated RNA demonstrated that the increased CAT production and transcription were accompanied by increased genome replication. Since both transcription and replication were affected, it is likely that mechanisms common to both pathways, such as initiation, are involved. However, it is unknown whether mRNAs are transcribed primarily from the encapsidated T7-generated template or from additional template amplified through replication. In the first case, transcription would be relatively independent of replication, while in the latter case, variations in replication would influence the amount of template available for transcription. Indeed, if replicated genome provides the main source of template for transcription, it is possible that the observed difference in transcription merely reflects the elevated levels of replication caused by the attenuated polymerases.

How the relatively high levels of RNA synthesis catalyzed by the L proteins of attenuated and vaccine strains of MV might affect the viral phenotype is not clear. The biologic equivalent of enhanced reporter gene expression in the minigenome replication assay would be increased production of viral RNA and proteins in vivo. Increased production of viral antigens during the early stages of infection may improve the stimulation of an antiviral immune response, possibly through enhanced antigen presentation by major histocompatibility complexes on infected cells (17), resulting in more rapid clearance of the attenuated viruses. Elevated RNA synthesis may also raise the amount of double-stranded RNA within the cell, resulting in more-efficient establishment of an antiviral state (5, 13). For example, MV infection induces the chemokine RANTES, which attracts monocytes and T cells during inflammation. RANTES induction is dependent on MV transcription, and a positive correlation was found between the extent of MV transcription and chemokine induction (26). Further exploration of the effect of increased viral RNA synthesis on pathogenicity requires the construction of chimeric MVs and their analysis in vivo.

All of the wild-type viruses used in this study were passaged in Vero cells, and therefore, their N, P, and L proteins may contain amino acid substitutions that resulted from host cell adaptation. Takeda et al. (42) reported that adaptation to Vero cells selected for mutations in the *L* and *P* genes, which led to decreased pathogenicity in cynomolgus monkeys. However, the amino acid substitutions those authors found in the L protein were not consistent with substitutions found in other Vero cell-adapted *L* gene sequences (2). A similar study reported amino acid substitutions in the *P* gene, but not the *L* gene, as a result of Vero cell adaptation (43). Therefore, it appears that Vero cell adaptation does not necessarily involve amino acid changes in the L protein, although the existence of such changes in the viruses used in this study cannot be ruled out.

The sequences of the *P* genes of Ne, Cam-70, Il-1, and Gam provide additional support for the finding that the P protein is among the least conserved of all MV proteins (1). Variability is more prevalent in the amino-terminal 231 aa, which constitute the region shared by P and V. The higher level of conservation of the carboxyl-terminal 276 aa is consistent with the proposed model for location of the major protein interaction sites, namely, the P-P, P-L, and P-N binding sites, in the unique part of P (23, 16). By analogy to the Sendai virus P protein, only one putative P-N binding site may be located in the extreme amino terminus of the MV P protein (8). We found no evidence of the previously described changes in the *P* gene that were suggested to be a consequence of adaptation to Vero cells (42, 43).

It has been proposed that adaptation to semipermissive cell lines, such as avian cells in the case of MV vaccines, initially selects for mutations that would improve the interaction of viral proteins with cellular factors and then requires second-site mutations in other viral proteins or *cis*-acting regions of the viral genome to ensure optimal cooperation among the different components of the viral replication apparatus (29). In the experiments reported here, coexpression of homologous N and P proteins did not increase the activities of the wild-type L proteins to those of the attenuated or vaccine viruses. Therefore, our results showed no evidence of coevolution of the N and P proteins with the L protein. Of course, the possibility remains that second-site mutations occurred in other MV proteins or noncoding regions of the genome. By using a similar minigenome replication assay, Reutter et al. recently demonstrated that CAT activity could be modulated by coexpression of P proteins from different viruses (34). This effect was due to differential inhibition of transcription by the C proteins translated from the *P* mRNAs. When the authors used *P* clones in which the *C* reading frame had been silenced, as we did in this study, all of the P proteins supported similar levels of transcription.

The *cis*-acting signals for transcription, replication, and encapsidation are located at the termini of the minigenome (40). Nucleotides 26 and 42 of the genomic leader were found to be A and G, respectively, in EdmBil and CAM-70, while the leaders of non-genotype A wild-type viruses consistently contain U at both positions (Liu, unpublished). Incorporation of the 26U and 42U substitutions into the minigenome did not increase the activity of wild-type polymerases; instead, it considerably reduced polymerase activity. It should be pointed out

that while the leader sequences of all three wild-type viruses in this study carry the 26U and 42U substitutions, only the Ne leader is identical to that of the puc26U42U construct. The leader sequences of Il-1 and Gam contain additional nucleotide changes (Liu, unpublished). While our results show that a genomic leader more closely resembling the natural leader of the wild-type viruses did not improve the activity of wild-type L proteins, a more thorough analysis of the promoter functions in recombinant MV is necessary to fully address this point.

Our findings demonstrate a functional difference between polymerase proteins of attenuated and wild-type viruses and suggest that mutations in the L protein may contribute to the attenuated phenotype of MV vaccine strains. Further analysis of the roles of individual amino acid changes in the context of a whole virus will be facilitated by the ability to insert different *L* genes into the infectious clone.

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